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# Identification of the two-phase mechanism of arachidonic acid regulating inflammatory prostaglandin E2 biosynthesis by targeting COX-2 and mPGES-1

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ABSTRACT

Through linking inducible cyclooxygenase (COX)-2 with microsomal prostaglandin E2 ($PGE_2$) synthase-1 (mPGES-1), a Single-Chain Enzyme Complex (SCEC, COX-2-10aa-mPGES-1) was engineered to mimic a specific inflammatory $PGE_2$ biosynthesis from omega-6 fatty acid, arachidonic acid (AA), by eliminating involvements of non-inducible COX-1 and other $PGE_2$ synthases. Using the SCEC, we characterized coupling reactions between COX-2 and mPGES-1 at 1:1 ratio of inflammatory $PGE_2$ production. AA demonstrated two phase activities to regulate inflammatory $PGE_2$ production. In the first phase (<2 μM), AA was a COX-2 substrate and converted to increasing production of $PGE_2$. In the second phase with a further increased AA level (2–10 μM), AA bound to mPGES-1 and inhibited the $PGE_2$ production. The SCEC study was identical to the co-expression of COX-2 and mPGES-1. This was further confirmed by using mPGES-1 and $PGH_2$ as a direct enzyme target and substrate, respectively. Furthermore, the carboxylic acid group of AA binding to R67 and R70 of mPGES-1 was identified by X-ray structure-based docking and mutagenesis. mPGES-1 mutants, R70A, R70K, R67A and R67K, lost 40–100% binding to [$^{14}$C]-AA. To conclude, a cellular model, in which AA is involved in self-controlling initial initiating and later resolving inflammation by its two phase activities, was discussed.



## 1. Introduction

Three $PGE_2$ synthases, microsomal prostaglandin E2 ($PGE_2$) synthase (mPGES)-1, mPGES-2 and cytosolic $PGE_2$ synthase (cPGES), have been cloned [1–4]. mPGES-1 is the major enzyme involved in producing inflammatory $PGE_2$ through instantly accepting the inducible cyclooxygenase (COX)-2-produced $PGH_2$ as a substrate. In addition, we have recently identified that COX-2 and mPGES-1 have a complex-like configuration anchored on the cellular ER membrane to effectively catalyze arachidonic acid (AA) to $PGE_2$ causing inflammation [5]. Thus, it is reasonable to posit that giving more omega-6 polyunsaturated fatty acid (PUFA), such as linoleic acid (precursor of AA), would advance tissue inflammation by acting on the COX-2/mPGES-1 system to produce more inflammatory $PGE_2$. However, recent animal and clinical trial studies have indicated that increasing daily consumption of omega-6 PUFA to at least 5–10% of total dietary intake likely decreases the risk of coronary heart disease (CHD) [6]. The molecular mechanisms behind the results are poorly understood.

Considering the major omega-6 PUFA is linoleic acid (consisting of 80–90% of the PUFA diet), which is converted into AA *in vivo*, investigation of AA metabolites in cells would further uncover the molecular mechanisms of the PUFA effects on reducing CHD. Three upstream enzymes, COX, lipoxygenase and cytochrome P450, are known to use AA as a substrate [7]. In a COX pathway, AA is metabolized into prostanoids, including prostacyclin ($PGI_2$), thromboxane A2 ($TXA_2$), $PGE_2$ and others, to maintain healthy physiological functions [8]. However, in inflammatory conditions

*Abbreviations:* 10aa, 10 amino acid residues; AA, arachidonic acid; CHD, coronary heart disease; COX-1 and -2, respectively, cyclooxygenase-1 and -2; cPGES, cytosolic PGES; EET, epoxyeicosatrienoic acid; HPLC, high performance liquid chromatography; HEK, human embryonic kidney; mPGES-1 and -2, respectively, microsomal PGE synthase-1 and -2; NSAIDs, nonsteroidal anti-inflammatory drugs; PUFA, polyunsaturated fatty acid; $PGI_2$, prostacyclin or prostaglandin I2; $PGE_2$, prostaglandin E2; $PGH_2$, prostaglandin H2; PDB, Protein Data Bank; SCEC, Single-chain Enzyme Complex; $TXA_2$, thromboxane A2.

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(such as vascular inflammation, arthritis and some cancers), AA is metabolized into a biased PG production toward $PGE_2$ to mediate the pathological processes, while reducing the amount of other prostanoids [9,10]. In lipoxygenase and cytochrome P450 pathways, AA is metabolized into leukotrienes and epoxyeicosatrienoic acids (EETs), having an impact on mediating asthma and vascular protection, respectively [11]. However, the COX pathway seems to be a dominated AA metabolism, observed by the inhibition of COXs by non-steroidal anti-inflammatory drugs (NSAIDs) that leads to a dramatic increase in cellular AA concentration [12]. Thus, characterizing the molecular and cellular activities of AA in detail shall be useful to further understand the cellular effects of omega-6 PUFAs on inflammatory diseases.

In the past, AA activity was studied under the co-expression condition of inducible COX-2 and mPGES-1 [13,14]. The uneven co-expression of COX-2 and mPGES-1, and the involvement of the other two $PGE_2$ synthases, cPGES and mPGES-2, became the limitation for quantifying how exact AA binding behaves from one molecule of COX-2 to one mPGES-1, and also the limitation for studying whether AA regulates the $PGE_2$ production in addition to being used as a substrate for COX-2. In this study, using our engineered Single-Chain Enzyme Complex (SCEC), COX-2-10aa-mPGES-1, which possesses three catalytic domains in a single polypeptide chain, we were able to limit the involvement of non-inducible $PGE_2$ synthases, cPGES and mPGES-2, to characterize AA metabolites during cellular inflammation. It led us to find the concentration-dependent of two-phase mechanism of AA, regulating cellular inflammatory $PGE_2$ biosynthesis as a COX-2 substrate and an mPGES-1 inhibitor at molecular and structural levels. The study has provided novel mechanisms to explain that a higher intake of omega-6 PUFAs could be even more beneficial to reduce vascular inflammation and CHD, which is highly interesting to public and medical communities in general.

## 2. Material and methods

### 2.1. Materials

HEK293 cell line was purchased from ATCC (Manassas, VA). Dulbecco's modified Eagle's medium, Geneticin, and Antibiotic-Antimycotic (100X) for culturing the cell lines and lipofectamine 2000 for transfecting cDNAs were purchased from Invitrogen (Carlsbad, CA). Fetal Bovine Serum (FBS) was from Life Technologies (Grand Island, NY). [$^{14}$C]-AA was purchased from PerkinElmer (Waltham, MA). [$^{3}$H]-$PGH_2$ and the primary antibodies for human COX-2 and mPGES-1 were purchased from Cayman Chemical Company (Ann Arbor, MI). The primary antibody of β-actin was purchased from Cell Signaling Technology (Danvers, MA). Primers for mPGES-1 mutants were purchased from Sigma-Aldrich, Inc (Staint Louis, MO). pcDNA™3.1 (+) was purchased from Invitrogen Corporation (Carlsbad, CA).

### 2.2. Establishing HEK293 cell lines expressing recombinant enzymes

The cDNA of the SCEC was previously reported from our group [15]. Briefly, HEK293 cells were transfected with the purified cDNA of the recombinant synthases (co-transfection with COX-2 and mPGES-1, transfection with COX-2-10aa-mPGES-1, mPGES-1, or mPGES-1 mutants) by the Lipofectamine 2000 method, following the manufacturer's instructions (Invitrogen). Approximately 48 h after transfection, HEK-COX-2/mPGES-1 cells were harvested for further enzyme assays. For the stable expression, the cells were incubated with culture medium (10% FBS, 1% Antibiotic-Antimycotic (100X) in DMEM containing geneticin (G418) 12 μL/mL).

### 2.3. Enzyme activity determination of HEK-COX-2/mPGES-1, HEK-COX-2-10aa-mPGES-1 and HEK-mPGES-1 using high performance liquid chromatography (HPLC)

Activities of the synthases were determined by monitoring metabolites of [$^{14}$C]-AA for HEK-COX-2/mPGES-1, HEK-COX-2-10aa-mPGES-1, and [$^{3}$H]-$PGH_2$ for HEK-mPGES-1. [$^{14}$C]-AA or [$^{3}$H]-$PGH_2$ was added to the harvested cells (about $2.2 \times 10^6$ cells) in a total reaction volume of 0.2 mL, and incubated at room temperature for 5 min. For the competition assays between the hot [$^{3}$H]-$PGH_2$ and the cold substrate $PGH_2$ (Cold $PGH_2$) or AA, 0.0125 μCi of [$^{3}$H]-$PGH_2$ and increasing concentration of cold $PGH_2$ or AA were added to the harvested cells. The reaction was stopped by adding 0.2 mL of Buffer A (containing 0.1% acetic acid and 35% acetonitrile). Subsequent to centrifugation at 13000 rpm for 10 min, the supernatant was collected and loaded onto a reverse phase C18 column (Varian Microsorb-MV 100-5, 4.6 mm × 250 mm). The sample was run with the Buffer A with a gradient from 35 to 100% of acetonitrile for 40 min with a flow rate 1.0 mL/min. The metabolite profile was obtained directly by a flow scintillation analyzer (Packard 150TR).

### 2.4. Docking of AA or $PGH_2$ with mPGES-1

The preparation, protomol generation and docking were performed using SYBYL-X 2.1 (Certara). A crystal structure of mPGES-1 (PDB # 4AL0) was used as a docking model. The structures of AA and $PGH_2$ were downloaded from PubChem (AA: PubChem CID # 466880, $PGH_2$: PubChem CID # 445049), and prepared by using the Concord program, computing charges (Huckel), and minimizing energy. The protomol was generated by a residues method. The active site residues were chosen based on the crystal structure information [16] with a threshold factor of 0.5 Å and a bloat of 0 Å. Docking was carried out with Surflex-Dock Geom (SFXC) mode [17–20].

### 2.5. Site-directed mutageneses of mPGES-1 mutants

cDNAs of the mPGES-1 mutants (R67A, R67K, R70A and R70K) were constructed using a standard PCR method (Fig. 1A) as

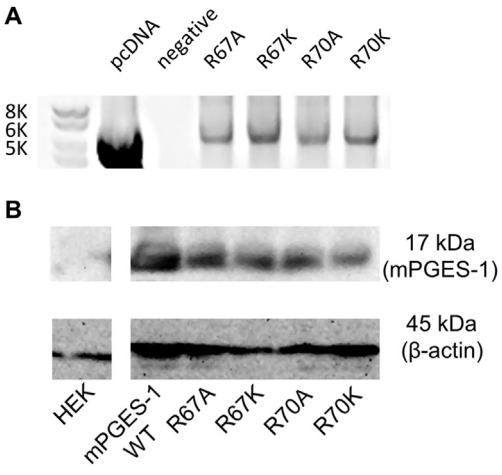


**Fig. 1.** A) Agarose gel electrophoresis of PCR for constructing cDNAs of mPGES-1 mutants. pcDNA: pcDNA3.1 (+)/mPGES-1 wild type. B) Western blot for mPGES-1 wild type and mutants expressed in HEK293. Untransfected HEK293 cells were used as a control.